

CUT&Tag-direct with CUTAC V.3



Steven Henikoff¹, Jorja Henikoff¹, Kami Ahmad¹

¹Fred Hutchinson Cancer Research Center

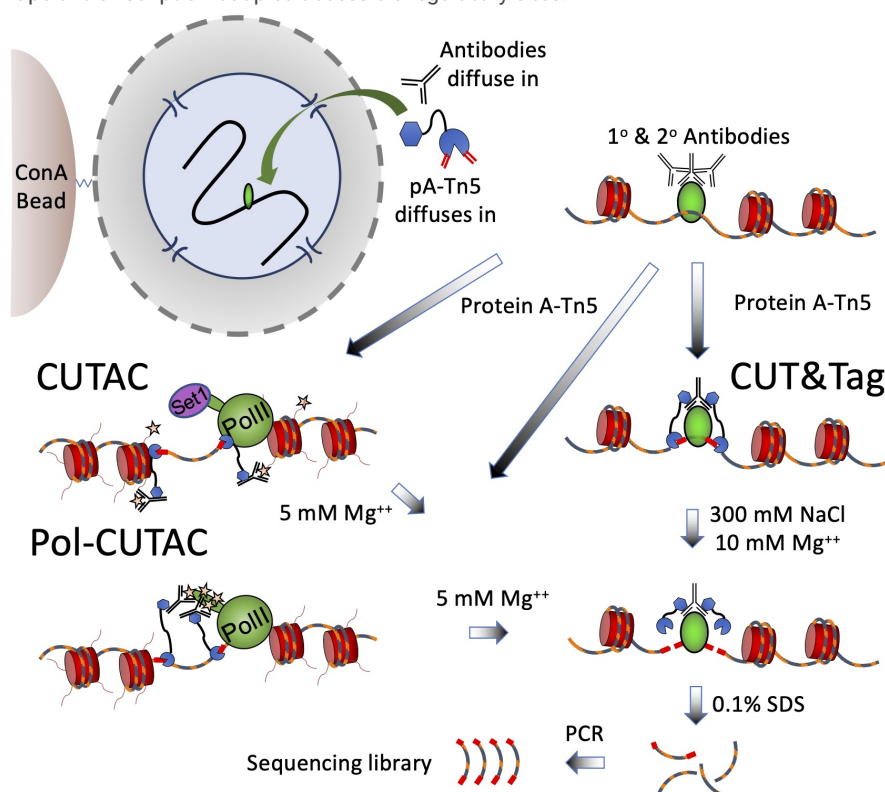


Steven Henikoff
Fred Hutchinson Cancer Research Center

bqwvmxe6

ABSTRACT

We previously introduced Cleavage Under Targets & Tagmentation (CUT&Tag), an epigenomic profiling method in which antibody tethering of the Tn5 transposase to a chromatin epitope of interest maps specific chromatin features in small samples and single cells. We have since adapted CUT&Tag to also map chromatin accessibility by simply modifying the Tn5 activation conditions when using histone H3K4me2, H3K4me3 or Serine-5-phosphorylated RNA Polymerase II (RNAPII) antibodies. Using these antibodies, tagmentation of accessible DNA sites is redirected to produce chromatin accessibility maps with exceptionally high signal-to-noise and resolution. All steps from nuclei to amplified sequencing-ready libraries are performed in single PCR tubes in the laboratory or on a home workbench, so that chromatin accessibility maps can be produced in parallel with CUT&Tag maps of other epitopes using a single protocol and analysis pipeline. Tethering of Tn5 to the initiation form of RNAPII at promoters and enhancers or to their flanking H3K4-methylated nucleosomes thus provides chromatin accessibility maps of transcription-coupled accessible regulatory sites.



Scheme for CUT&Tag, CUTAC and Pol-CUTAC. CUT&Tag-direct is performed in situ in single PCR tubes with Concanavalin A (ConA) bead-bound nuclei that remain intact throughout the protocol during successive liquid changes, incubations and washes, 12 cycles of PCR amplification and one SPRI bead clean-up. CUTAC is performed identically except that low-salt conditions are used for tagmentation. CUTAC maps accessible sites near H3K4me2/3-marked (starred) nucleosome tails, which are methylated by the conserved Set1 lysine methyltransferase. The complex that includes Set1 associates with the initiation form of RNAPII, which is heavily phosphorylated on Serine-5 of the heptameric C-terminal domain repeat units on the largest RNAPII subunit (RNAPIIS5). In Pol-CUTAC, pA-Tn5 is anchored directly to RNAPIIS5 phosphates (starred). Whereas CUT&Tag is suitable for any chromatin epitope, CUTAC is specific for H3K4me2 and H3K4me3 and Pol-CUTAC for RNAPIIS5P. The only other difference between the protocols is that tagmentation is performed in the presence of 300 mM NaCl for CUT&Tag and in a low ionic strength buffer for both versions of CUTAC.

EXTERNAL LINK

<https://doi.org/10.7554/eLife.63274>

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Steven Henikoff, Jorja G. Henikoff, Hatice S Kaya-Okur, Kami Ahmad (2020) **Efficient transcription-coupled chromatin accessibility mapping in situ** *eLife* 9:e63274, DOI: 10.7554/eLife.63274.

EXTERNAL LINK

<https://doi.org/10.7554/eLife.63274>

PROTOCOL INFO

Steven Henikoff, Jorja Henikoff, Kami Ahmad . CUT&Tag-direct with CUTAC. **protocols.io**
<https://protocols.io/view/cut-amp-tag-direct-with-cutac-bqwvmxe6>

MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

Steven Henikoff, Jorja G. Henikoff, Hatice S Kaya-Okur, Kami Ahmad (2020) **Efficient transcription-coupled chromatin accessibility mapping in situ** *eLife* 9:e63274, DOI: 10.7554/eLife.63274.

KEYWORDS

CUT&Tag, CUTAC, epigenetics, chromatin profiling

CREATED

Dec 20, 2020

LAST MODIFIED

Feb 05, 2021

PROTOCOL INTEGER ID

45749

GUIDELINES

Based on <https://www.protocols.io/view/bench-top-cut-amp-tag-z6hf9b6>.

MATERIALS TEXT



A home workbench for CUT&Tag. A photo of the authors' home laundry room counter setup used for all experiments presented using this protocol. A typical experiment begins by mixing cells with activated ConA beads in 32 single PCR tubes, with all liquid changes performed on the magnet stands. The only tube transfer is removal of the purified sequencing-ready libraries from the SPRI beads to fresh tubes for Tapestation analysis and DNA sequencing. The total time from thawing frozen nuclei until elution from SPRI beads is ~8 hours.

- Chilling device (e.g. metal heat blocks on ice or cold packs in an ice cooler)
- Pipettors (e.g. Rainin Classic Pipette 1 mL, 200 µL, 20 µL, 10 µL)
- Disposable tips (e.g. Rainin 1 mL, 200 µL, 20 µL)
- Disposable centrifuge tubes for reagents (15 mL or 50 mL)
- Standard 1.5 ml microfuge tubes
- 0.5 ml maximum recovery PCR tubes (e.g. Fisher cat. no. 14-222-294)
- Frozen native or cross-linked nuclei suspension (e.g. human K562 or H1 cells) prepared as described in the Appendix

(Steps 48-60, cite: [Kaya-Okur et al. \(2020\) Nature Protocols](#)).

- Concanavalin A (ConA)-coated magnetic beads (Bangs Laboratories, ca. no. BP531)
- Strong magnet stand (e.g. Miltenyi Macsimag separator, cat. no. 130-092-168)
- Vortex mixer (e.g. VWR Vortex Genie)
- Mini-centrifuge (e.g. VWR Model V)
- PCR thermocycler (e.g. BioRad/MJ PTC-200)
- Distilled, deionized or RNase-free H₂O (dH₂O e.g., Promega, cat. no. P1197)
- 1 M Hydroxyethyl piperazineethanesulfonic acid pH 7.9 (HEPES (K+); Sigma-Aldrich, cat. no. H3375)
- 1 M Manganese Chloride (MnCl₂; Sigma-Aldrich, cat. no. 203734)
- 1 M Calcium Chloride (CaCl₂; Fisher, cat. no. BP510)
- 1 M Potassium Chloride (KCl; Sigma-Aldrich, cat. no. P3911)
- Roche Complete Protease Inhibitor EDTA-Free tablets (Sigma-Aldrich, cat. no. 5056489001)
- 1 M Hydroxyethyl piperazineethanesulfonic acid pH 7.5 (HEPES (Na+); Sigma-Aldrich, cat. no. H3375)
- 5 M Sodium chloride (NaCl; Sigma-Aldrich, cat. no. S5150-1L)
- 2 M Spermidine (Sigma-Aldrich, cat. no. S0266)
- 0.5 M Ethylenediaminetetraacetic acid (EDTA; Research Organics, cat. no. 3002E)
- 200X Bovine Serum Albumen (BSA, NEB, cat no. B9001S)
- Antibody to an epitope of interest. Because in situ binding conditions are more like those for immunofluorescence (IF) than those for ChIP, we suggest choosing IF-tested antibodies if CUT&RUN/Tag-tested antibodies are not available
- CUTAC control antibody to RNA Polymerase II Phospho-Rpb1 CTD Serine-5 phosphate (PolII S5P) or histone H3K4me2. We have obtained excellent results with these rabbit monoclonal antibodies: Cell Signalling Technology Phospho-Rpb1 CTD (Ser5), CST #13523 (D9N5I) and Epicypher H3K4me2 #13-0027.
- Secondary antibody, e.g. guinea pig α -rabbit antibody (Antibodies online cat. no. ABIN101961) or rabbit α -mouse antibody (Abcam cat. no. ab46540)
- Protein A/G-Tn5 (pAG-Tn5) fusion protein loaded with double-stranded adapters with 19mer Tn5 mosaic ends Epicypher cat. no. 15-1117.
- 1 M Magnesium Chloride (MgCl₂; Sigma-Aldrich, cat. no. M8266-100G)
- 1 M [tris(hydroxymethyl)methylamino]propanesulfonic acid (TAPS) pH 8.5 (with NaOH)
- 1,6-hexanediol (Sigma-Aldrich cat. no. 240117-50G)
- N,N-dimethylformamide (Sigma-Aldrich cat. no. D-8654-250mL)
- NEBNext 2X PCR Master mix (ME541L)
- PCR primers: 10 μ M stock solutions of i5 and i7 primers with unique barcodes [Buenrostro, J.D. et al. Nature 523:486 (2015)] in 10 mM Tris pH 8. Standard salt-free primers may be used. We do not recommend Nextera or NEBNext primers.
- 10% Sodium dodecyl sulfate (SDS; Sigma-Aldrich, cat. no. L4509)
- 10% Triton X-100 (Sigma-Aldrich, cat. no. X100)
- SPRI paramagnetic beads (e.g. HighPrep PCR Cleanup Magbio Genomics cat. no. AC-60500)
- 10 mM Tris-HCl pH 8.0
- Ethanol (Decon Labs, cat. no. 2716)

SAFETY WARNINGS

There are no hazardous materials or dangerous equipment used in this protocol, however appropriate lab safety training is recommended.

BEFORE STARTING

Prepare and cryopreserve nuclei (STEPS 50-62 at the lab)

Prepare reagents (STEP 1, OK at home)

REAGENT SETUP (for up to 16 samples)

- 1 **Binding buffer** Mix 200 μ L 1 M HEPES-KOH pH 7.9, 100 μ L 1 M KCl, 10 μ L 1 M CaCl₂ and 10 μ L 1 M MnCl₂, and bring the final volume to 10 mL with dH₂O. Store the buffer at 4 °C for up to several months.

Wash buffer Mix 1 mL 1 M HEPES pH 7.5, 1.5 mL 5 M NaCl, 12.5 μ L 2 M spermidine, bring the final volume to 50 mL with dH₂O, and add 1 Roche Complete Protease Inhibitor EDTA-Free tablet. Store the buffer at 4 °C for up to 2 days.

Antibody buffer Mix 5 μ L 200X BSA with 1 mL Wash buffer and chill on ice. BSA is present in some but not all antibody solutions, and 0.1% BSA in this buffer helps to prevent bead loss during later steps.

300-wash buffer Mix 1 mL 1 M HEPES pH 7.5, 3 mL 5 M NaCl and 12.5 μ L 2 M spermidine, bring the final volume to 50 mL with dH₂O and add 1 Roche Complete Protease Inhibitor EDTA-Free tablet. Store at 4 °C for up to 2 days.

CUT&Tag Tagmentation buffer Mix 1 mL 300-wash buffer and 10 μL 1 M MgCl_2 (to 10 mM).

CUTAC Tagmentation buffer Mix 197 μL dH_2O , 2 μL 1 M TAPS pH 8.5 and 1 μL 1 M MgCl_2 (10 mM TAPS, 5 mM MgCl_2).

CUTAC-hex Tagmentation buffer Mix 97 μL dH_2O , 100 μL 20% (w/v) 1,6-hexanediol, 2 μL 1 M TAPS pH 8.5 and 1 μL 1 M MgCl_2 (10 mM TAPS, 5 mM MgCl_2).

TAPS wash buffer Mix 1 mL dH_2O , 10 μL 1 M TAPS pH 8.5, 0.4 μL 0.5 M EDTA (10 mM TAPS, 0.2 mM EDTA)

0.1% SDS Release solution Mix 10 μL 10% SDS and 10 μL 1 M TAPS pH 8.5 in 1 mL dH_2O

0.67% Triton neutralization solution Mix 67 μL 10% Triton-X100 + 933 μL

Prepare Concanavalin A-coated beads (15 min)

- 2 Resuspend and withdraw enough of the ConA bead slurry such that there will be 3-4 μL for each final sample of up to ~50,000 mammalian cells. Transfer the ConA bead slurry into 1 mL Binding buffer in a 1.5 mL tube

This protocol has been used for up to 16 samples (60 μL beads) in 1 mL or 32 samples (120 μL beads) in 2 mL Binding buffer (in a 2 mL tube).

- 3 Mix by pipetting. Place the tube on a magnet stand to clear (~1 min).
- 4 Withdraw the liquid completely, and remove from the magnet stand. Add 1 mL Binding buffer and mix by pipetting.
- 5 Place on magnet stand to clear, withdraw liquid, and resuspend in 60 μL Binding buffer (for 3-4 μL per sample).

Bind nuclei to ConA beads (15 min)

- 6 Thaw a frozen aliquot of nuclei at room temperature, for example by placing in a 20 mL beaker of water.

The CUTAC control can use either native or lightly cross-linked nuclei, preferably prepared as described in the Appendix (Steps 50-62). Do not use whole cells, which require a detergent and may inhibit the PCR.

- 7 Transfer the thawed nuclei suspension in aliquots of no more than ~50,000 starting mammalian cells to each PCR tube and mix with 3-5 μL ConA beads in thin-wall 0.5 mL PCR tubes. Rotate at room temperature for 10 min.

Nuclei prepared according to the protocol in the Appendix (Steps 50-62) have been resuspended in Wash buffer. Beads can be added directly to the aliquot for binding and then transferred to PCR tubes such that no more than 5 μL of the original ConA bead suspension is present in each PCR tube for single-tube CUT&Tag.

Using more than ~50,000 mammalian nuclei or >5 μ L Con A beads per sample may inhibit the PCR.

- 8 Place the tubes on the magnet stand to clear and withdraw the liquid.

In low-retention PCR tubes, surface tension will cause bead-bound cells at this step to slide down to the bottom of the tube at this step, so to avoid losses here and below, set the pipettor to 5 μ L less than the liquid volume to be removed and use a careful second draw with a 20 μ L pipette tip to remove most of the last liquid without losing beads.

Bind primary antibody (1 hr)

- 9 Resuspend cells in 25 μ L per sample of Antibody buffer containing antibody (1:50-1:100) with gentle vortexing. For each CUTAC control sample, prepare a separate PolII Ser5P or H3K4me2 tube.

We use 1:50-1:100 antibody dilution by default or the manufacturer's recommended concentration for immunofluorescence.

The CUTAC control works best using either an RNA PolII CTD-phosphorylated antibody (Ser5P > Ser2P/Ser5P > Ser2P) or an α -H3K4me2 antibody. α -H3K4me3 also works but is less efficient and is depleted at enhancer sites. Several antibodies to other histone epitopes have been tested, including α -H3K4me1, α -H3K36me3, α -H3K27ac and α -H2A.Z but all have failed.

- 10 Place on a Rotator at room temperature and incubate 1-2 hr.

🕒 01:00:00

Volumes up to 50 μ L will remain in the tube bottom by surface tension during rotation.

After incubation, tubes can be held overnight at 4 °C.

The α -RNA PolII Ser5P and α -H3K4me2 antibodies used for both CUT&Tag and CUTAC serve as controls to evaluate success of the CUTAC procedure. An optional negative control is performed by omitting the primary antibody.

Bind secondary antibody (1 hr)

- 11 Place tubes on the magnet stand to clear and remove the liquid.

Protein in the antibody solution improves bead adherence to the tube wall, allowing for complete removal of liquid without dislodging the beads using two successive draws of a 20 µL pipettor set for maximum volume, being careful not to dislodge the beads by surface tension during the second draw.

- 12 Mix the secondary antibody 1:100 in Wash buffer and squirt in 25 µL per sample while gently vortexing to allow the solution to dislodge the beads from the sides.

The secondary antibody step is required for CUT&Tag to increase the number of Protein A binding sites for each bound antibody. We have found that without the secondary antibody the efficiency is very low.

- 13 Place the tubes on a Rotator and rotate at room temperature for 0.5-1 hr. 🕒 00:30:00

30m

- 14 After a quick spin (<500 x g or just enough to remove the liquid from the sides of the tube), place the tubes on the magnet stand to clear and withdraw the liquid with two successive draws using a 20 µL tip with the pipettor set for maximum volume.

- 15 With the tubes still on the magnet stand, carefully add 500 µL Wash buffer. The surface tension will cause the beads to slide up along the side of the tube closest to the magnet.

- 16 Slowly withdraw 470 µL with a 1 mL pipette tip without disturbing the beads.

To withdraw the liquid, set the pipettor to 470 µL, and keep the plunger depressed while lowering the tip to the bottom. The liquid level will rise to near the top completing the wash. Then ease off on the plunger until the liquid is withdrawn, and remove the pipettor. During liquid removal, the surface tension will drag the beads down the tube. A small drop of liquid that is left behind is removed in the next step.

- 17 After a quick spin replace the tubes on the magnet stand and withdraw the liquid with a 20 µL pipettor using multiple draws. Proceed immediately to the next step.

Bind pA-Tn5 adapter complex (1.5 hr)

- 18 Mix pAG-Tn5 pre-loaded adapter complex in 300-wash buffer following the manufacturer's instructions.

- 19 Squirt in 25 µL per sample of the pA-Tn5 mix while vortexing and invert by rotation to allow the solution to dislodge any remaining beads from the sides.

When using the recommended Macsimag magnet stand, dislodging the beads can be done by removing the plexiglass tube holder from the magnet, and with fingers on top to prevent the tubes from opening up or falling out, invert by rotating sharply a few times.

- 20 After a quick spin (<500 x g), place the tubes on a Rotator at room temperature for 1-2 hr. 🕒 01:00:00

1h

- 21 After a quick spin place the tubes on a magnet stand to clear and and withdraw the liquid with a 20 μ L pipettor using two successive draws.
- 22 With the tubes still on the magnet stand, add 500 μ L 300-wash buffer.
- 23 Slowly withdraw 470 μ L with a 1 mL pipette tip without disturbing the beads as in Step 16.
- 24 After a quick spin, replace the tubes on the magnet stand to clear and withdraw the liquid with a 20 μ L pipettor using multiple draws. Proceed immediately to Step 26. For a CUTAC sample proceed immediately to Step 25.

CUTAC Tagmentation (performed in parallel with other samples)

- 25 Resuspend the bead/nuclei pellet in 50 μ L CUTAC (5 mM $MgCl_2$, 10 mM TAPS) or CUTAC-hex tagmentation solution (5 mM $MgCl_2$, 10 mM TAPS, 10% 1,6-hexanediol) while vortexing or inverting by rotation to allow the solution to dislodge most or all of the beads as in Step 19. Place the tube(s) in a cold block and proceed to Step 27.

10% 1,6-hexanediol or N,N-dimethylformamide compete for hydrophobic interactions and result in improved tethered Tn5 accessibility and library yield.

CUT&Tag tagmentation and particle release (2.5 hr)

- 26 Resuspend the bead/nuclei pellet in 50 μ L tagmentation solution while vortexing or inverting by rotation to allow the solution to dislodge most or all of the beads as in Step 19.
- 27 After a quick spin (<500 x g), incubate at 37 °C for 1 hr (20 min for CUTAC) in a PCR cycler with heated lid. Hold at 8 °C.
🕒 01:00:00 🕒 00:20:00
- 28 Place tubes on the magnet stand and withdraw the liquid with a 20 μ L pipettor using multiple draws then resuspend the beads in 50 μ L TAPS wash and invert by rotation as in Step 19.
- 29 After a quick spin, replace tubes on the magnet stand, and withdraw the liquid with a 20 μ L pipettor using multiple draws.
- 30 Resuspend the beads in 5 μ L 0.1% SDS Release solution using a fresh 20 μ L pipette tip to dispense while wetting the sides of the tubes to recover the fraction of beads sticking to the sides.

Twirling the tube back and forth rapidly between thumb and finger will effectively wet the sides of the tube, followed by a quick spin to bring most of the beads to the bottom.

31 Incubate at 58 °C for 1 hr in a PCR cycler with heated lid to release pA-Tn5 from the tagmented DNA. ⌚ 01:00:00

PCR (1 hr)

32 To the PCR tube containing the bead slurry add 15 µL Triton neutralization solution + 2 µL of 10 µM Universal or barcoded i5 primer + 2 µL of 10 µM uniquely barcoded i7 primers, using a different barcode for each sample. Vortex on full and place tubes in metal tube holder on ice.

Indexed primers are described by Buenrostro, J.D. et al. Single-cell chromatin accessibility reveals principles of regulatory variation. Nature 523:486 (2015). We do not recommend Nextera or NEB primers.

33 Add 25 µL NEBnext (non-hot-start), vortex to mix, followed by a quick spin. Proceed immediately to the PCR.

34 Mix, quick spin and place in Thermocycler and begin cycling program with heated lid:

Cycle 1: 58 °C for 5 min (gap filling)

Cycle 2: 72 °C for 5 min (gap filling)

Cycle 3: 98 °C for 30 sec

Cycle 4: 98 °C for 10 sec

Cycle 5: 60 °C for 10 sec

Repeat Cycles 4-5 11 times

72°C for 1 min and hold at 8 °C

To minimize the contribution of large DNA fragments and excess primers, PCR should be performed for no more than 12-14 cycles, preferably with a 10 s 60-63 °C combined annealing/extension step.

The cycle times are based on using a conventional Peltier cycler (e.g., BioRad/MJ PTC200), in which the ramping times (3 °C/sec) are sufficient for annealing to occur as the sample cools from 98 °C to 60 °C. Therefore, the use of a rapid cycler with a higher ramping rate will require either reducing the ramping time or other adjustments to assure annealing.

Do not add extra PCR cycles to see a signal by capillary gel electrophoresis (e.g. Tapestation). If there is no nucleosomal ladder for the H3K27me3 positive control, you may assume that CUT&Tag failed, but observing no signal for a sparse chromatin protein such as a transcription factor is normal, and the barcoded sample can be concentrated for mixing with the pool of barcoded samples for sequencing. Extra PCR cycles reduce the complexity of the library and may result in an unacceptable level of PCR duplicates.

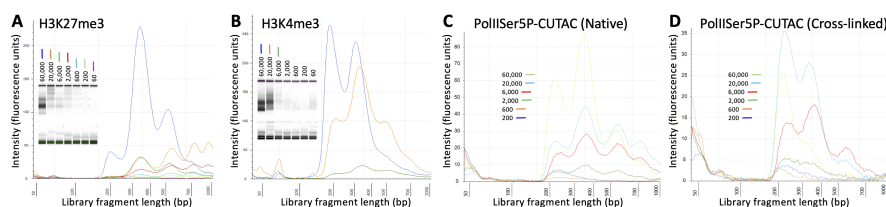
Post-PCR Clean-up (30 min)

35 After tubes have cooled, remove from the cycler and add 1.3 volume (65 µL) SPRI bead slurry, mixing by pipetting up and down.

- 36 Let sit at room temperature 5-10 min. ⌚ 00:05:00
- 37 Place on magnet a few minutes to allow the beads to clear before withdrawing the liquid. While still on the magnet stand add 200 μ L 80% ethanol.
- 38 Withdraw the liquid with a pipette to the bottom of the tube, and add 200 μ L 80% ethanol.
- 39 Withdraw the liquid and after a quick spin, remove the remaining liquid with a 20 μ L pipette. Do not air-dry the beads, but proceed immediately to the next step.
- 40 Remove from the magnet stand, add 22 μ L 10 mM Tris-HCl pH 8 and vortex on full. Let sit at least 5 min. ⌚ 00:05:00
- 41 Place on the magnet stand and allow to clear.
- 42 Remove the liquid to a fresh 1.5 ml tube with a pipette, avoiding transfer of beads.

Tapestation analysis and DNA sequencing (outsourcing)

- 43 Determine the size distribution and concentration of libraries by capillary electrophoresis using an Agilent 4200 TapeStation with D1000 reagents or equivalent.



CUT&Tag-direct and Pol-CUTAC for low cell numbers: At home: Frozen K562 cell nuclei (~50% recovery) were thawed and diluted into 50 μ L volumes of Wash buffer in PCR tubes, and 3 μ L of activated ConA beads was added to each. Successive incubations with primary antibody, secondary antibody, pA-Tn5 and Mg⁺⁺, followed by SDS release, amplification for 12 cycles, and SPRI bead cleanup in the same PCR tubes produced ready-to-sequence libraries from thawed nuclei in 8 hours. **(A)** H3K27me3, **(B)** H3K4me3, **(C)** PolII Ser5P-CUTAC (Native) **(D)** PolII Ser5P-CUTAC (Cross-linked). **At the lab:** 1/10th of each sample was analyzed by TapeStation. Nucleosomal ladders were detectable for intermediate and low numbers of cells. The barcoded libraries were mixed with others for paired-end PE25 sequencing on an Illumina 2-lane HiSeq2500 flowcell. Representative tracks for the cross-linked Pol-CUTAC samples are shown under Step 46.

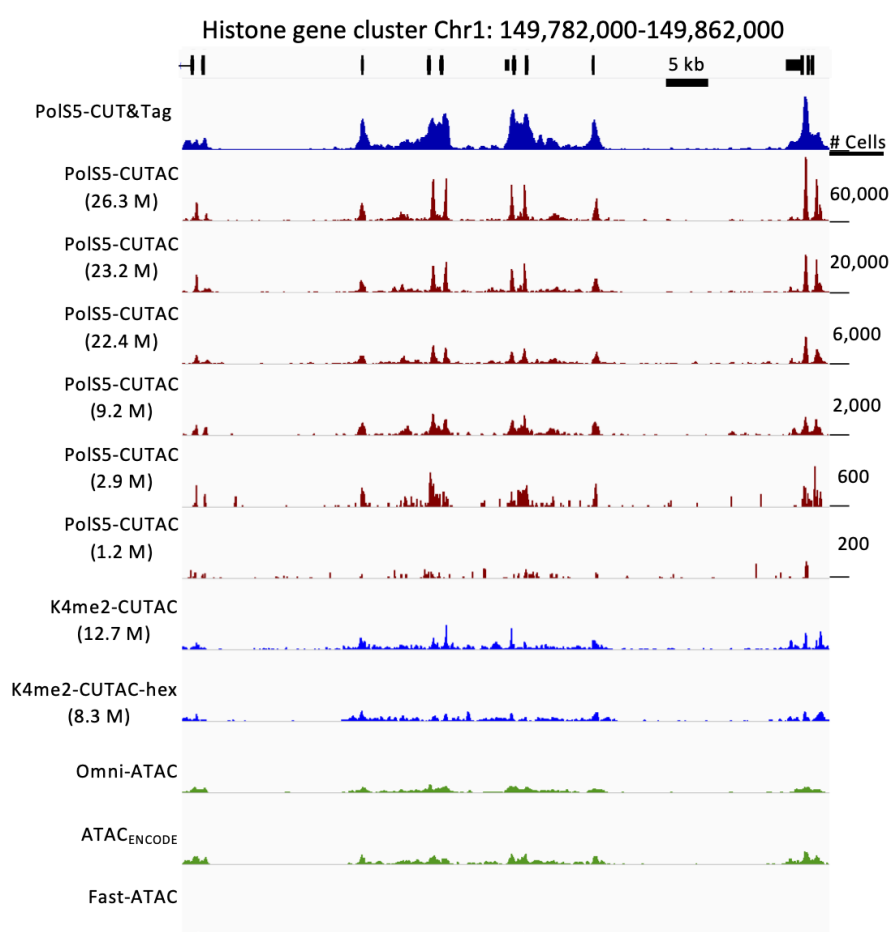
- 44 Mix barcoded libraries to achieve equal representation as desired aiming for a final concentration as recommended by the manufacturer. After mixing, perform an SPRI bead cleanup if needed to remove any residual PCR primers.
- 45 Perform paired-end Illumina sequencing on the barcoded libraries following the manufacturer's instructions. For maximum economy, paired-end PE25 is more than sufficient for mapping to large genomes.

Using paired-end 25x25 sequencing on a HiSeq 2-lane rapid run flow cell we obtain ~300 million total mapped reads, or ~3 million per sample when there are 96 samples mixed to obtain approximately equal molarity.

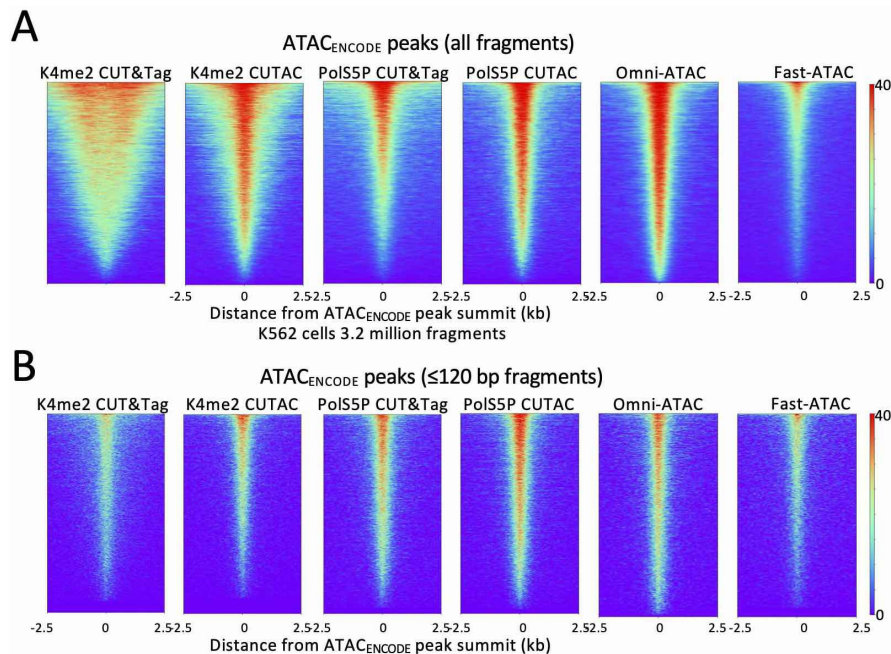
Data processing and analysis

- 46 We align paired-end reads to hg19 using Bowtie2 version 2.3.4.3 with options: `--end-to-end --very-sensitive --no-unal --no-mixed --no-discordant --phred33 -l 10 -X 700`. For mapping *E. coli* carry-over fragments, we also use the `--no-overlap --no-dovetail` options to avoid possible cross-mapping of the experimental genome to that of the carry-over *E. coli* DNA that is used for calibration. Tracks are made as bedgraph files of normalized counts, which are the fraction of total counts at each basepair scaled by the size of the hg19 genome.

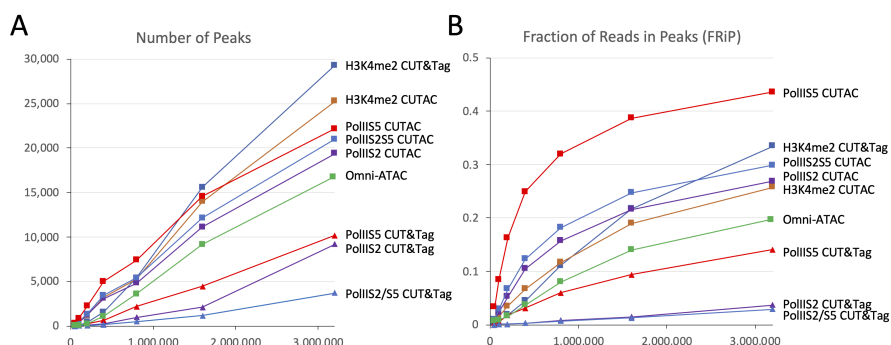
To calibrate samples in a series for samples done in parallel using the same antibody we use counts of *E. coli* fragments carried over with the pA-Tn5 the same as one would for an ordinary spike-in. Our [sample script in Github](#) can be used to calibrate based on either a spike-in or *E. coli* carry-over DNA.



Sensitive and precise accessible site mapping with Pol-CUTAC. Group autoscaled tracks show the Chromosome 1 histone gene cluster, with 12 small intronless genes expressed at high levels in all dividing cells. Whereas PolII Ser5P CUT&Tag (top) shows broad enrichment over each of the genes, the CUTAC protocol applied to the PolII Ser5P epitope (using lightly cross-linked K562 cells) yields sharp promoter delineation, better than H3K4me2 CUTAC \pm 1,6-hexanediol (blue) or ATAC-seq datasets (green). Numbers in parentheses are library sizes in millions of mapped paired-end reads estimated using Picard tools. Fast-ATAC is an improved version of ATAC-seq that reduces mitochondrial reads, and Omni-ATAC is an improved version that additionally improves signal-to-noise. ATAC_{ENCODE} is the current ENCODE standard. Datasets are available from GEO ([GSE158327](#))



Accessible DNA corresponds to binding sites of the initiating form of RNA Polymerase II (PolISer5P). **A)** Left to right: K4me2 CUT&Tag, K4me2 CUTAC, PolISer5 phosphate (PolIS5P) CUT&Tag, PolISer5P CUTAC, Omni-ATAC and Fast-ATAC datasets were downsampled to 3.2 million fragments and aligned over ATAC-seq peaks called using MACS2 on data generated by the ENCODE project (ATAC_ENCODE). **B)** Same as (A) except using only subnucleosome-sized fragments (≤ 120 bp). CUTAC PolISer5P sites are virtually indistinguishable from high-quality ATAC-seq data, directly demonstrating that ATAC-seq maps sites of the initiation form of RNAPII. ENCODE ATAC-seq fragments were downsampled to 3.2 million, ChrM (mitochondrial DNA) was removed and MACS2 was used to call peaks. Heatmaps are centered over ENCODE ATAC-seq peak summits and ordered by occupancy over the 5-kb span displayed. Fast-ATAC is an improved version of ATAC-seq that reduces mitochondrial reads, and Omni-ATAC is an improved version that additionally improves signal-to-noise. ATAC_ENCODE is the current ENCODE standard. Datasets are available from GEO ([GSE158327](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE158327))



Pol-CUTAC shows high sensitivity and specificity. Mapped fragments from the indicated K562 datasets were sampled and peaks were called using MACS2. **A)** Number of peaks and **B)** fraction of reads in peaks for CUT&Tag (triangles) and CUTAC (squares) profiles for H3K4me2, PolISer5 phosphate (initiation form), PolISer2 phosphate (elongation form), PolISer2/S5 phosphate and Omni-ATAC (green). Pol-CUTAC for PolIS5 phosphate shows the best sensitivity (most peaks at low sampling) and the best signal-to-noise (highest FRiP at all sampling levels). Tagmentation was for 10 min at 37 °C in CUTAC-tag buffer. Datasets are available from GEO ([GSE158327](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE158327)).

- 47 Our [CUT&Tag Data Processing and Analysis Tutorial](#) on Protocols.io provides step-by-step guidance for mapping and analysis of CUT&Tag sequencing data. Most data analysis tools used for ChIP-seq data, such as [bedtools](#), [Picard](#) and [deepTools](#), can be used on CUT&Tag data. Analysis tools designed specifically for CUT&RUN/Tag data include the [SEACR peak caller](#) also available as a [public web server](#) and [CUT&RUNTools](#).

For best Pol-CUTAC results, map ≤ 120 bp fragments, which enrich for fragments confined to accessible DNA and improve peak-calling.

See [Efficient chromatin accessibility mapping *in situ* by nucleosome-tethered tagmentation](#) for examples of

Appendix: Prepare and (optionally) lightly fix nuclei and cryopreserve (1 hr in the lab)

48 Prepare NE1 buffer: Mix 1 ml 1M HEPES-KOH pH 7.9, 500 μ L 1M KCl, 12.5 μ L 2 M spermidine, 500 μ L 10% Triton X-100, and 10 ml glycerol in 38 ml dH₂O, and add 1 Roche Complete Protease Inhibitor EDTA-Free.

Prepare Wash buffer Mix 1 mL 1 M HEPES pH 7.5, 1.5 mL 5 M NaCl, 12.5 μ L 2 M spermidine, bring the final volume to 50 mL with dH₂O, and add 1 Roche Complete Protease Inhibitor EDTA-Free tablet.

Other reagents:

- Phosphate-buffered saline (1X PBS, 10X stock solution from Fisher cat. no. BP3994)
- 16% (w/v) formaldehyde (10 x 1 ml ampules, Thermo-Fisher ca. no. 28906)
- 1.25 M glycine
- Dimethyl sulfoxide (DMSO; Sigma-Aldrich cat. no. D4540)

49 Harvest fresh culture(s) in a conical centrifuge tube (15 ml or 50 ml) at room temperature and count cells.

50 Centrifuge 3 min 600 x g in a swinging bucket rotor at room temperature and drain liquid.

51 Resuspend in 1 volume PBS at room temperature while pipetting. For fixed cells skip to Step 58.

52 Centrifuge 3 min 600 x g in a swinging bucket rotor at room temperature and drain liquid.

53 Resuspend in 1/2 volume (relative to starting culture) ice-cold NE1 buffer with gentle vortexing. Let sit on ice 10 min.

54 Centrifuge 4 min 1300 x g at 4 °C in a swinging bucket rotor and drain liquid by pouring off and inverting onto a paper towel for a few seconds.

55 Resuspend in 1/2 volume of PBS. For unfixed nuclei, skip to Step 60.

56 While gently vortexing add 16% formaldehyde to 0.1% (e.g., 62 μ L to 10 ml) and incubate at room temperature for 2 minutes.

Based on: Oh KS, Ha J, Baek S, Sung MH. XL-DNase-seq: improved footprinting of dynamic transcription factors *Epigenetics Chromatin* 2019;12(1):30.

Light fixation reduces the tendency of cells or nuclei to clump in the Dig-300 buffer, but can interfere with binding of some antibodies, reducing yield. Clumping can also be reduced for fixed and unfixed nuclei prepared using this

protocol by omitting digitonin from all steps. We have not observed any difference in CUT&Tag results for nuclei prepared using Triton-X100 with or without digitonin.

- 57 Stop cross-linking by addition of 1.25 M glycine to twice the molar concentration of formaldehyde (e.g., 600 μ L to 10 ml).
- 58 Centrifuge 4 min 1300 x g at 4 °C and drain liquid by pouring off and inverting onto a paper towel for a few seconds.
- 59 Resuspend in Wash buffer to a concentration of ~1 million cells per ml. Check nuclei using ViCell or cell counter slide.
- 60 Nuclei may be slowly frozen by aliquoting 900 μ L into cryogenic vials containing 100 μ L DMSO, mixed well, then placed in a Mr. Frosty container filled to the line with isopropanol and placed in a -80 °C freezer overnight then stored at -80 °C long term.

We have found that excellent results are obtained using native or cross-linked cells even after being stored in the freezer compartment of a side-by-side refrigerator for >6 months.